JOURNAL OF BACTERIOLOGY, July, 1966
Vol. 92, No. 1


# Protection of Monkeys Against Experimental Shigellosis with a Living Attenuated Oral Polyvalent Dysentery Vaccine

SAMUEL B. FORMAL, T. H. KENT, H. C. MAY, A. PALMER, S. FALKOW, AND E. H. LABREC
*Walter Reed Army Institute of Research, Washington, D.C., and National Institutes of Health, Bethesda, Maryland*

Received for publication 14 March 1966

ABSTRACT

FORMAL, SAMUEL B. (Walter Reed Army Institute of Research, Washington, D.C.), T. H. KENT, H. C. MAY, A. PALMER, AND E. H. LABREC. Protection of monkeys against experimental challenge with a living attenuated oral polyvalent dysentery vaccine. J. Bacteriol. **91**:17–22. 1966.—Virulent strains of *Shigella flexneri* 1b, *S. flexneri* 3, and *S. sonnei* I were mated with an Hfr strain of *Escherichia coli* K-12, and hybrids were selected for the xylose marker. One hybrid strain of each of the serotypes was chosen for study of their biological characteristics. Their capacity to cause a fatal enteric infection in starved guinea pigs was reduced, they failed to cause dysentery when fed to monkeys, they caused keratoconjunctivitis in the guinea pig eye, and they penetrated HeLa cells. Two doses of a polyvalent oral vaccine composed of *S. flexneri* 1b, 2a, and 3, and *S. sonnei* I hybrid strains were fed to groups of monkeys at an interval of 4 to 7 days, and they, together with controls, were challenged 10 days after the last dose with one or another of the virulent parent dysentery strains. A significant degree of protection was afforded in all vaccinated groups with the exception of one group challenged with *S. flexneri* 6, a component not included in the vaccine. When animals were challenged with virulent *S. flexneri* 2a 1 month after oral vaccination, they were also protected. The vaccine produced a rise in serum antibody, but we were not able to detect coproantibody in saline extracts of feces from animals which received the vaccine.

We previously have shown that a strain of *Shigella flexneri* 2a lost its capacity to cause a fatal enteric infection in starved guinea pigs when, by recombination, it incorporated into its genome the genetic material between the rhamnose-xylose region of an Hfr strain of *Escherichia coli* (1). Such a hybrid strain retained its ability to invade tissue, but appeared to be unable to multiply in the intestinal mucosa (2). A single oral dose of $5 \times 10^{10}$ cells of the hybrid strain sufficed to render monkeys resistant to experimental oral challenge with the virulent parent strain of *S. flexneri* 2a (3).

There are over 30 different serotypes of dysentery bacilli, and it is not at all certain whether hybrid strains of others would possess properties similar to those of our *S. flexneri* 2a hybrid strain. The purpose of this communication is to describe the characteristics of hybrid strains of *S. flexneri* 1b, *S. flexneri* 3, and *S. sonnei* I, and to present the results of studies in which a living polyvalent oral vaccine was used to protect monkeys against experimental challenge.

## MATERIALS AND METHODS

*Cultures.* *S. flexneri* 1b strain 1Z, *S. flexneri* 2a strain 2457T, *S. flexneri* 3 strain J17B, *S. flexneri* 6 strain CCHO60, and *S. sonnei* I strain 53G were all isolated from human beings with bacillary dysentery. *Escherichia coli* Hfr strain W1895 and *E. coli-S. flexneri* 2a hybrid strain X16 have been described previously (2, 3; Formal et al., *in press*).

*Mating procedures.* The methods used to obtain *E. coli-Shigella* hybrid strains were similar to those already described (1, 2).

*Biological tests.* Tests for the ability of the various strains to invade HeLa cells, to produce keratoconjunctivitis, to fatally infect guinea pigs, to multiply in the small intestine of the starved guinea pig, to cause dysentery in monkeys, and to protect monkeys against experimental challenge have been described previously (2, 3, 4).

*Serological tests.* The passive hemagglutination test was used to detect serum and coproantibody

levels. The methods employed have been described (Formal et al., *in press*). In the present study, the procedure for coporantibody determinations was modified to the extent that the stool extracts were diluted in 15% human serum albumin instead of saline.

## RESULTS

*Genetic studies.* *S. flexneri* 1b strain 1Z, *S. flexneri* 3 strain J17B, and *S. sonnei* I strain 53G were mated with Hfr *E. coli* K-12 strain W1895 and plated on minimal medium containing xylose but lacking methionine. Clones from the *E. coli-Shigella* matings were purified, and their biochemical patterns were determined. After preliminary testing, a single hybrid strain from each mating was selected for further studies. The genetic characteristics of the parent and hybrid strains are presented in Table 1.

*Studies in guinea pigs.* The ability of the hybrids and wild-type shigellae to cause a fatal enteric infection in starved guinea pigs is compared in Table 2. The virulence of the hybrids was markedly reduced in each case. By use of the fluorescent-antibody technique, each of the hybrid strains was shown to retain the property of being able to penetrate the intestinal epithelium and to reach the lamina propria. This resulted in a mild acute inflammatory reaction in the ileal mucosa. The ability of the parent and hybrid strains to grow in the small intestine of the experimentally infected guinea pig was next compared. The parent strains remained in the small intestine in large numbers over the course of the experiment, whereas the hybrids were rapidly cleared from the small intestine (Fig. 1). Thus, the hybrid strains of *S. flexneri* 1b, *S. flexneri* 3, and *S. sonnei* I behaved in the starved guinea pig model in a manner similar to our *S. flexneri* 2a hybrid strain X16 (2).

*Keratoconjunctivitis studies.* All of the parent shigella strains caused a severe keratoconjunctivitis in guinea pigs. The hybrids also produced keratoconjunctivitis. The reactions produced by the hybrid strains of *S. flexneri* 2a and *S. flexneri* 3 were similar to those evoked by the parent strains; however, the hybrid strains of *S. flexneri* 1b and *S. sonnei* I tended to be milder, in that a reduced amount of purulent exudate was observed.

*Tissue culture studies.* The hybrid strains were tested for their ability to enter into HeLa cells (4). Each hybrid retained the capacity to enter the HeLa cells. *S. flexneri* 1b and *S. flexneri* 3 hybrid strains which had acquired pili tended to stick to the HeLa cells in addition to penetrating them.

*General characteristics of the hybrids.* The colonial appearance of *S. flexneri* 1b, *S. flexneri* 3, and *S. sonnei* I hybrid strains was smooth, and dense saline suspensions of cells of the three hybrid strains failed to agglutinate when heated for 2 hr in a boiling-water bath. The *S. flexneri* 1b and *S. flexneri* 3 hybrids grew sparsely on nutrient agar (Difco), but Brain Heart Infusion (BHI) Agar (Difco) supported good growth. The *S. sonnei* I hybrid grew well on nutrient agar and, like its parent strain, dissociated to form phase II colonies. The phase II colonies from the hybrid *S. sonnei* I strain retained the hybrid biochemical pattern, and were avirulent in that they failed to cause reaction in either the guinea pig ileum or in the guinea pig eye and did not penetrate HeLa cells.

Each of the hybrid strains agglutinated (slide test) in its respective typing serum, and the *S. flexneri* 1b and *S. flexneri* 3 hybrids gave positive reactions in *S. flexneri* group factor 6 serum. The hybrid shigella strains were used to adsorb sera prepared against wild-type *S. flexneri* 1b, *S.*

TABLE 1. *Genetic characteristics of parent and hybrid strains*[a]

| Organism | Strain | Auxotrophic character (met) | Utilization of | | | | | | Indole production | Pili antigen | Mating polarity |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | lac | ara | rha | xyl | mal | fuc | | | |
| *Shigella flexneri* 1b | 1Z | + | − | − | − | − | − | − | − | − | $F^-$ |
| *S. flexneri* 1b hybrid | 1ZX13 | + | − | − | + | + | − | − | − | + | $F^-$ |
| *S. flexneri* 2a | 2457T | + | − | − | − | − | − | − | − | − | $F^-$ |
| *S. flexneri* 2a, hybrid | 245TX16 | + | − | + | + | + | + | − | − | − | $F^-$ |
| *S. flexneri* 3 | J17B | + | − | − | − | − | − | − | + | − | $F^-$ |
| *S. flexneri* 3, hybrid | J17BX19 | + | + | + | − | + | + | − | + | + | $F^-$ |
| *S. sonnei* I | 53G | + | − | + | + | − | + | − | − | − | $F^-$ |
| *S. sonnei* I, hybrid | 53GX7 | + | − | + | + | + | + | − | + | − | $F^-$ |
| *Escherichia coli* K-12 | W1895 | − | + | + | + | + | + | + | + | + | Hfr |

[a] The following abbreviations are used: met, methionine; lac, lactose; ara, arabinose; rha, rhamnose; xyl, xylose; mal, maltose; fuc, fucose; + = synthesis or utilization; − = not synthesized or utilized; $F^-$ recipient; Hfr, high frequency of recombination donor.

*flexneri* 3, and *S. sonnei* I strains. Hybrid *S. flexneri* 1b and *S. flexneri* 3 strains reduced the respective wild-type homologous titer from 1:5,120 to a weak reaction in 1:80, and hybrid *S. sonnei* I strain from 1:1,280 to less than 1:80. This may be indicative of minor antigenic changes in the *S. flexneri* 1b and 3 hybrid strains; each serum (2 ml of a 1:40 dilution) was adsorbed twice with $10^{11}$ viable cells of each hybrid strain, which usually is sufficient to remove agglutinins completely.

*Reactions in monkeys.* A single dose consisting of $5 \times 10^{10}$ cells (grown on BHI Agar) suspended in 20 ml of BHI broth was fed to groups of eight monkeys, and the animals were observed for 1 week. None of the animals developed diarrhea or dysentery. Similar preparations were administered to groups of five or six monkeys; these animals were killed and autopsied 48 hr after challenge. No gross changes were seen in the colon or other organs. The only abnormality noted microscopically consisted of a very mild, patchy colitis with polymorphonuclear leukocytes in the lamina propria and a few scattered crypt abscesses.

*Polyvalent vaccine studies.* A polyvalent vaccine consisting of hybrid *S. flexneri* 1b strain 1Z × 13, *S. flexneri* 2a strain M22-18 × 16 (2, 3, Formal et al., *in press*), *S. flexneri* 3 strain J17B × 19, and *S. sonnei* I strain 53G × 7 was tested for its ability to protect monkeys against experimental challenge. Two doses of this vaccine were fed at intervals of 4 to 7 days; a dose consisted of $2 \times 10^{10}$ viable cells of each of the component strains suspended in 20 ml of BHI broth. Of 212 animals which received the polyvalent vaccine, 29 had diarrhea. The diarrhea usually occurred after the first vaccine dose, and stools usually returned to normal within the next 24 hr.

Shedding of the vaccine strains after oral feeding differed from monkey to monkey. From some animals, we failed to isolate the vaccine strain after its administration. It may be noted here that these animals still responded with a rise in serum antibody levels (see below). Some animals shed one or another of the component strains, and others shed two, three, or all of the hybrids. In most cases, the components were isolated for no longer than 4 days after the last vaccine dose; however, an occasional animal intermittently excreted a hybrid strain for longer periods.

TABLE 2. *Deaths observed in starved guinea pigs after the oral administration of parent and hybrid strains of Shigella*[a]

| Strain | Deaths/total | |
|---|---|---|
| | Parent | Hybrid |
| *S. flexneri* 1b..... | 36/71 | 3/66 |
| *S. flexneri* 3..... | 53/90 | 1/94 |
| *S. sonnei* 1..... | 20/31 | 5/38 |

[a] Animals were starved for 4 days; the challenge was $5 \times 10^7$ to $1 \times 10^8$ viable bacteria; 1 ml of tincture of opium was injected intraperitoneally after challenge.

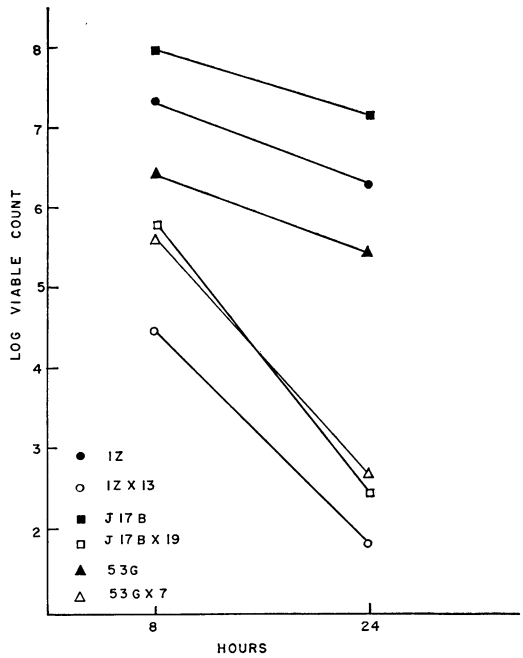


FIG. 1 *In vivo growth of the parent and hybrid strains of shigellae in the small intestine of guinea pigs starved 4 days and then fed approximately* $5 \times 10^7$ *viable cells of one of the strains. The points on the curves represent the geometric mean of the number of viable organisms recovered from the entire ground-up small intestine of four animals. 1Z = Shigella flexneri 1b; 1Z × 13 = S. flexneri 1b hybrid; J17B = S. flexneri 3; J17B × 19 = S. flexneri 3 hybrid; 53G = S. sonnei I; 53G × 7 = S. sonnei I hybrid.*

The passive hemagglutination test was used to determine the serum antibody titer in a total of 38 animals. Serum specimens were obtained before and 5 days after the last vaccine dose. All of the animals responded to one or another of the vaccine components with fourfold or greater rises in serum antibody titers. In most cases, rises in titer to all components were observed. In those instances where responses were not detected, the preimmunization antibody level was usually high. The pattern of this response is summarized in Table 3.

Coproantibody studies were carried out on 11

TABLE 3. *Serological response[a] of 38 monkeys which received two oral doses of a living attenuated polyvalent dysentery vaccine and were bled 5 days after the last dose was administered*

| Antigen | Reciprocal of prevaccine titer | Reciprocal of postvaccine titer | | | | | | | No. of animals with | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 15 | 30 | 60 | 120 | 240 | 480 | 960 | No rise | Twofold rise | Fourfold or greater rise |
| *Shigella flexneri* 1b | <15 (1)[b] | | | | | 1[b] | | | | | |
| | 15 (9) | | | 3 | 2 | 3 | 1 | | | | |
| | 30 (7) | | | 1 | 4 | 2 | | | 6 | 8 | 24 |
| | 60 (10) | | | 1 | 3 | 3 | 1 | 2 | | | |
| | 120 (5) | | | | 1 | 2 | 2 | | | | |
| | 240 (4) | | | | | 2 | 2 | | | | |
| | 480 (2) | | | | | | 2 | | | | |
| *S. flexneri* 2a | <15 (20) | 3 | 4 | 3 | 6 | 4 | | | | | |
| | 15 (7) | | 2 | 1 | 3 | 1 | | | | | |
| | 30 (6) | | | 3 | 2 | | | 1 | 2 | 10 | 26 |
| | 60 (2) | | | | 1 | 1 | | | | | |
| | 120 (3) | | | | 2 | 1 | | | | | |
| *S. flexneri* 3 | <15 (4) | | | 2 | 1 | 1 | | | | | |
| | 15 (8) | | 1 | 4 | 1 | 2 | | | | | |
| | 30 (10) | | 1 | 1 | 2 | 6 | | | 6 | 8 | 24 |
| | 60 (8) | | | 1 | 3 | 4 | | | | | |
| | 120 (5) | | | | 3 | 1 | 1 | | | | |
| | 240 (2) | | | | | 1 | 1 | | | | |
| | 480 (1) | | | | | | | 1 | | | |
| *S. sonnei* I | <15 (16) | | 1 | 4 | 5 | 4 | 1 | 1 | | | |
| | 15 (10) | | | 2 | 3 | 2 | | | | | |
| | 30 (5) | | | | 2 | 2 | 1 | | 1 | 0 | 37 |
| | 60 (5) | | | | | 4 | 1 | | | | |
| | 120 (1) | | | | | | 1 | | | | |
| | 480 (1) | | | | | | 1 | | | | |

[a] Passive hemagglutination test was used to determine antibody levels.
[b] Number of animals with corresponding pre- or postvaccine titer.

animals which received two doses of the vaccine 1 week apart. Saline extracts of feces were obtained before the vaccine was fed and 5 days after the last vaccine dose. We failed to observe any consistent rise in passive hemagglutination titer to any of the component strains of the vaccine.

Five monkeys autopsied 48 hr after challenge with the polyvalent vaccine had no gross lesions. Microscopically, four animals had patchy areas of mild colitis consisting mainly of small crypt abscesses. The fifth animal had patchy areas of slightly more severe colitis with some changes in surface cells as well as larger crypt abscesses. However, this reaction would be considered mild compared with the lesions produced by virulent shigellae (Formal et al., *in press*).

The results of protection tests in which groups of monkeys receiving the polyvalent vaccine and control animals were challenged with $5 \times 10^{10}$ viable cells of virulent *S. flexneri* 1b, *S. flexneri* 2a, *S. flexneri* 3, or *S. sonnei* I 10 days after the last vaccine dose are summarized in Table 4. In each instance, the monkeys receiving the vaccine experienced significantly less shigellosis than those animals in the control group. The symptoms in the vaccinated monkeys, when they were observed, tended to be milder than those in the control group, and often consisted of a single diarrheal stool. Only 3 cases of classical dysentery (diarrheal stool with blood and mucus) occurred in the vaccinated monkeys, whereas 35 control animals exhibited this syndrome. None of the animals receiving the vaccine died after the challenge. Eight control monkeys succumbed. It should be noted that in most instances the monkeys were treated with antibiotics whenever blood was observed in the stools. In the experiment where deaths were observed, treatment was withheld from both the vaccinated and control animals. In one experiment, the animals were challenged with *S. flexneri* 6, which was

not a component of the vaccine. There was no evidence of protection.

Two experiments were conducted in which vaccinated and control monkeys were challenged with *S. flexneri* 2a 1 month after the vaccine group received its last dose. In the first experiment, the vaccine was monovalent and consisted of $5 \times 10^{10}$ cells per dose of the hybrid strain of *S. flexneri* 2a; in the second experiment, the polyvalent vaccine was used. The results indicate that the animals were still protected 1 month after vaccination (Table 5). All animals which developed bloody stools in these two experiments were given antibiotic therapy; however, treatment was not started soon enough to save four of the control animals which died in the second experiment.

## DISCUSSION

In previous studies, we demonstrated that the virulence of a single strain of *S. flexneri* 2a was markedly reduced for starved guinea pigs and monkeys after acquisition by mating of genetic material between the *rha*$^+$–*xyl*$^+$ region of the *E. coli* K-12 genome (1). The present work suggests that dysentery strains of other serotypes also lose much of their capacity to cause a fatal enteric infection in starved guinea pigs and to cause serious disease in monkeys when similarly mated with *E. coli*. All of the hybrids caused reactions in the guinea pig eye, but this test revealed differences in the various hybrid strains. The hybrid strains of *S. flexneri* 1b and *S. sonnei* I evoked reactions which tended to be milder than those of *S. flexneri* 2a or 3, even though all of the parent dysentery strains produced severe keratoconjunctivitis. However, all of the hybrid strains did produce an inflammatory reaction in the intestinal tracts of starved guinea pigs and of monkeys, and all penetrated HeLa cells.

With four hybrid strains of dysentery at hand, all of which qualitatively had similar biological activity, a decision had to be made concerning how they would be tested for their ability to protect monkeys against experimental challenge. We first considered testing the ability of each hybrid strain to confer resistance, but rejected this approach because of the number of animals involved. Instead, we decided to pool the four strains and to test the capacity of this oral polyvalent vaccine to confer protection against each of the four virulent parental dysentery serotypes. Under the conditions of our test system, the polyvalent vaccine effectively protected monkeys against the various challenge strains. Diarrhea in vaccinated animals when it occurred tended to be mild, and classical dysentery was observed in only 3 of 254 vaccinated animals which had

TABLE 4. *Signs of illness in vaccinated and control monkeys challenged 10 days after the last vaccine dose with virulent strains of Shigella*[a]

| Challenge strain | Vaccine group | | Total ill/total challenged | Control group | | | *P*, vaccine vs. control |
|---|---|---|---|---|---|---|---|
| | No. with diarrhea | No. with dysentery | | No. with diarrhea | No. with dysentery | Total ill/total challenged | |
| *S. flexneri* 1b | 1 | 0 | 1/55 | 20 | 4 | 24/58 | $<1 \times 10^{-8}$ |
| *S. flexneri* 2a | 2 | 1 | 3/40 | 4 | 16 (8 died) | 20/50 | $3 \times 10^{-4}$ |
| *S. flexneri* 3 | 5 | 1 | 6/54 | 10 | 7 | 17/58 | $<1 \times 10^{-2}$ |
| *S. sonnei* I | 10 | 1 | 11/63 | 31 | 8 | 39/59 | $<4 \times 10^{-8}$ |
| *S. flexneri* 6 | 6 | 5 | 11/17 | 9 | 5 | 14/17 | $>8 \times 10^{-1}$ |

[a] The vaccine contained *Escherichia coli-Shigella* hybrid strains of *S. flexneri* 1b, *S. flexneri* 2a, *S. flexneri* 3, and *S. sonnei* I. The animals were fed two doses of the vaccine at intervals of 4 to 7 days.

TABLE 5. *Signs of illness in vaccinated and control monkeys challenged 1 month after the last vaccine dose with virulent Shigella flexneri 2a*

| Vaccine[a] | Vaccine group | | | Control group | | | *P*, vaccine vs. control |
|---|---|---|---|---|---|---|---|
| | No. with diarrhea | No. with dysentery | Total ill/total challenged | No. with diarrhea | No. with dysentery | Total ill/total challenged | |
| Monovalent | 2 | 0 | 2/19 | 1 | 7 | 8/23 | $<10^{-2}$ |
| Polyvalent | 6 | 0 | 6/23 | 3 | 13 (4 died) | 16/21 | $<10^{-4}$ |

[a] The monovalent vaccine consisted of an *E. coli-S. flexneri* 2a hybrid strain; the polyvalent vaccine contained *E. coli-Shigella* hybrid strains of *S. flexneri* 1b, *S. flexneri* 2a, *S. flexneri* 3, and *S. sonnei* I. The animals were fed two doses of the vaccine 7 days apart.

been challenged with either *S. flexneri* 1b, 2a, or 3, or *S. sonnei* I. Most of our protection results come from experiments in which animals were challenged 10 days after the last vaccine dose was administered. In two experiments, monkeys challenged 1 month after vaccination with either monovalent *S. flexneri* 2a vaccine or the polyvalent preparation were still protected.

The question remains concerning the relative protective potency of a monovalent preparation as opposed to the polyvalent product. The two experiments in which animals were challenged 1 month after receiving either the hybrid *S. flexneri* 2a strain or the polyvalent vaccine indicate that differences in protection, if they do exist, are small. However, with the available, admittedly crude, assay procedures which we necessarily must presently employ, the numbers of monkeys which would be required to detect small differences in potency of two different products would be too large, and we must be satisfied for the time being in determining whether a given preparation protects or whether it does not.

The polyvalent vaccine evoked a detectable serum antibody response in virtually all animals. In most cases, rises in titer were observed to all the vaccine components. Adsorption studies were not carried out to determine whether the rise was due to response to type-specific or group-specific antigen or to both. We were unable to detect a consistent rise in antibody levels to any of the vaccine components in extracts of feces from animals receiving the polyvalent vaccine.

In this study and in our previous work (3; Formal et al., *in press*), we have demonstrated that monkeys can be effectively protected against the symptoms usually seen after experimental challenge with virulent dysentery bacilli. The problem still faces us as to whether protection will be observed in situations where infection occurs under natural conditions. We hope to answer this question presently.

## LITERATURE CITED

1. FALKOW, S., H. SCHNEIDER, L. S. BARON, AND S. B. FORMAL. 1963. Virulence of *Escherichia-Shigella* genetic hybrids for the guinea pig. J. Bacteriol. **86**:1251–1258.
2. FORMAL, S. B., E. H. LABREC, T. H. KENT, AND S. FALKOW. 1965. Abortive intestinal infection with an *Escherichia coli-Shigella flexneri* hybrid strain. J. Bacteriol. **89**:1374–1382.
3. FORMAL, S. B., E. H. LABREC, A. PALMER, AND S. FALKOW. 1965. Protection of monkeys against experimental shigellosis with attenuated vaccines. J. Bacteriol. **90**:63–68.
4. LABREC, E. H., H. SCHNEIDER, T. J. MAGNANI, AND S. B. FORMAL. 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. **88**:1503–1518.